Plant Physiol. (1996) 111: 159–168

A Thiol Protease and an Anionic Peroxidase Are Induced by Lowering Cytokinins during Callus Growth in *Petunia*

Colette Tournaire, Serguei Kushnir, Guy Bauw, Dirk Inzé, Bernard Teyssendier de la Serve, and Jean-Pierre Renaudin*

Laboratoire de Biochimie et Physiologie Végétales, Institut National de la Recherche Agronomique/Ecole Nationale Supérieure Agronomique/Centre National de la Recherche Scientifique, F34060 Montpellier Cedex 1, France (C.T., B.T.d.l.S., J.-P.R.); Laboratorium voor Genetica, Universiteit Gent, B9000 Gent, Belgium (S.K., G.B.); and Laboratoire associé de l'Institut National de la Recherche Agronomique (Institut National de la Recherche Agronomique, France), Universiteit Gent, B9000 Gent, Belgium (D.I.)

We previously identified a group of proteins that increase early in *Petunia hybrida* calli subcultured on a low-cytokinin medium, unlike the calli subcultured on a high-cytokinin medium. The calli on the low-cytokinin medium do not regenerate (J.-P. Renaudin, C. Tournaire, B. Teyssendier de la Serve [1991] Physiol Plant 82: 48–56). Two of these proteins, P21 and P17, have been identified by peptide sequencing and cloned. P21 is highly homologous to a group of thiol proteases, including barley aleurain, rice oryzain γ, Arabidopsis SAG2, and mammalian cathepsin H. P17 is highly homologous to a group of anionic peroxidases from potato and tomato. A study of their expression in two *P. hybrida* lines, PC6 and St40, which differ in their ability to regenerate, showed that the genes for P21 and P17 are differentially expressed depending on the type and the age of the organ, with the highest expression in senescing leaves and in aged calli. The data are in favor of these genes being associated with an early step of senescence, which may be due, in part, to a reduction in total cytokinin. The two *Petunia* lines are, thus, functionally different concerning the action of cytokinin in two developmental phenomena: in vitro organogenesis and senescence.

In vitro organogenesis is a complex, developmental pathway of plant tissues under the control of a large range of effectors, including, notably, growth substances, nutrients, and light (Thorpe, 1980). The duration of the biological response, spanning days and even weeks, and the paucity of genetic studies have notably contributed to our limited understanding of the control of in vitro organogenesis. Cytokinins are considered key regulators of the formation of vegetative buds in vitro, both when they are added in vitro (Skoog and Miller, 1957) and when they are synthesized endogenously (Estruch et al., 1991; Li et al., 1992). Our knowledge of cytokinin function still lags behind that of the other plant growth substances, although some progress has been made in this area recently (Binns, 1994).

We have developed a biological system to search for molecular markers of cytokinin action during in vitro organogenesis (Renaudin et al., 1990). Microcalli grown from *Petunia hybrida* mesophyll protoplasts were induced to regenerate buds, roots, or further calli according to the absolute concentrations of auxin and cytokinin added to the culture medium, and the effect of cytokinin on vegetative bud formation was studied (Renaudin et al., 1990; Traas et al., 1990). The continuous presence of cytokinin was required for 2 weeks, until bud primordia were formed. Vegetative buds became visible at the end of the 3rd week of culture. The growth of calli during the first 2 weeks occurred to the same extent when the cytokinin concentration was lowered 10-fold, but primordium induction and vegetative bud regeneration were triggered only by the highest cytokinin concentration.

2D SDS-PAGE patterns of total protein of *Petunia* calli cultured for various times in the presence of a high (i.e. leading to bud formation) or a low (i.e. leading only to callus growth) concentration of cytokinin were analyzed as a first step to understanding the cytokinin-controlled change in the developmental program. Five groups of proteins were identified that showed quantitative variation during culture (Renaudin et al., 1991). One group of four proteins accumulated early, during the 2nd week of culture, only in calli cultured in the presence of the lowest cytokinin concentration. They remained nearly absent in calli grown at the highest cytokinin concentration. No morphological difference was visible at that time between calli grown at either cytokinin concentration.

It was decided to characterize further the proteins of the latter group, since they seemed to be induced by the relative reduction of cytokinin. We report here the identification of two of the four proteins as a thiol protease and an anionic peroxidase. They have been cloned, and their expression, during callus regeneration and in whole plants, was assessed at the RNA level. The significance of the coordinated expression of these proteins in cytokinin-deprived, nonregenerating callus will be discussed.

MATERIALS AND METHODS

Plant Material and in Vitro Culture

Petunia hybrida protoplasts isolated from leaf mesophyll of axenically propagated plants of the lines PC6 and St40

* Corresponding author; e-mail renaudin@msdos.ensam.inra.fr; fax 33–67–52–57–37.

Abbreviations: 2D, two-dimensional; 2iP, δ^2-isopentenyladenine.

were cultured for 1 week to induce the first division (Renaudin et al., 1990). Two- to four-cell colonies were then plated at low cell density, 100 cells mL^{-1}, in liquid medium C (Muller et al., 1983) in the presence of 1 μM naphtalene acetic acid and 1 μM N^6-benzylaminopurine. After a growth period of 21 d, the microcalli were transferred to a solid bud-regeneration medium (medium B) containing Murashige and Skoog's basal salts (Murashige and Skoog, 1962), Morel and Wetmore's vitamins (Morel and Wetmore, 1951), 20 g L^{-1} Suc, 53 g L^{-1} mannitol, 0.7% (w/v) agarose (Seakem LE, FMC Products, Rockland, ME), 3 mM (2-[*N*-morpholino]ethanesulfonic acid)-KOH, pH 5.8, 5 μM IAA, and 25 μM 2iP. The calli were cultured at 24°C in the light (9 μmol m^{-2} s^{-1} photon flux, 16-h photoperiod). Regeneration was not induced if cytokinin concentration was 2.5 μM instead of 25 μM 2iP in medium B.

Protein Analysis by 2D-PAGE and Microsequencing

Protein extraction, analytical 2D-PAGE, and densitometric measurements of protein spots by image analysis were performed as previously reported (Renaudin et al., 1991). For preparative 2D-PAGE, thioglycolic acid was added to the protein solubilization buffer and to the upper buffer during the 2D SDS-PAGE at a final concentration of 15 mM. Ampholyte concentration was increased in IEF preparative gels, compared with analytical gels, to 1.2%, pH 5–7 (Bio-Rad), 1.2%, pH 6–8 (Pharmacia), and 0.6%, pH 3–10 (Pharmacia). IEF and SDS gels were subjected to electrophoresis before loading the samples, for 30 min at 300 V and 2 h at 100 V, respectively. Preparative 3 × 135 mm IEF gels were loaded with 500 to 900 μg of protein. After staining with Coomassie blue (R250) and destaining, the proteins were electroblotted to a polyvinylidene fluoride membrane (Immobilon, Millipore) in a buffer of 50 mM boric acid, 50 mM Tris-HCl for 2O h at 35 V. The spots corresponding to proteins P21 and P17 (Renaudin et al., 1991) were incubated for 30 min at room temperature in 800 μL of methanol containing 0.2% (w/v) PVP. The membranes were rinsed four times with distilled water and once with 0.1 M Tris-HCl, pH 8.5. The proteins were partly digested with 4 μg of porcine pancreatic trypsin (Sigma) in 200 μL of 0.1 M Tris-HCl, pH 8.5, at 37°C for 5.5 h. The peptides recovered from the membrane were separated on a C18 reverse-phase HPLC column (Vydac, Separations Group, Hesperia, CA) with a linear gradient from 0 to 70% acetonitrile in 0.1% TFA at a rate of 1 mL min^{-1}. Peptides were detected by absorption at 215 nm, collected, and concentrated to 50 μL in vacuo. The concentrated fractions were used directly for sequencing by automated Edman degradation using a gas-phase sequencer (Applied Biosystems) (Bauw et al., 1987).

RNA and DNA Extraction and Hybridization

Total RNA was extracted from various parts of *Petunia* plants and calli by phenol-SDS extraction (Teyssendier de la Serve and Jouanneau, 1979). Total RNA was denaturated in 50% formamide and 2.2 M formaldehyde for 5 min at 65°C and separated in 1.25% agarose gels containing formaldehyde (Sambrook et al., 1989). DNA was isolated from plant leaves (Davies et al., 1980). After 12 h of hydrolysis by restriction enzymes, the DNA fragments were separated on 0.8% agarose gels in 90 mM Tris-borate, 2 mM Na_2-EDTA (Sambrook et al., 1989).

After blotting onto membranes (Hybond N, Amersham), RNA and DNA were hybridized with probes ^{32}P-labeled by random priming. Hybridization was performed in 0.9 M NaCl, 50 mM NaH_2PO_4, 5 mM Na_2-EDTA, 10% dextran sulfate, 1% *N*-lauroylsarcosine, and 50% formamide. The probes were the PCR-amplified DNA corresponding to P17 (PePer1, 615 bp) and to P21 (a 350-bp cDNA also used for the cloning of PeTh3) and a partial cDNA for tobacco 25 S rRNA (NT7, 191 bp, J.P. Renaudin, unpublished data). Hybridization was performed for 15 h at 42°C. The washings were done successively for 20 min at 50°C in 0.45 M NaCl, 45 mM Na_3-citrate (3× SSC), and 1% *N*-lauroylsarcosine; for 20 min at 50°C in 1× SSC and 1% *N*-lauroylsarcosine; and for 30 min at 60°C in 0.1× SSC.

cDNA Amplification and Cloning

Degenerate oligonucleotides were made from the partial sequences of two peptides from P21: 5′-GA(A/G)GCIGGI-ATIGTI(A/T)(G/C)ICCIGTIAA-3′ corresponding to the sense orientation of EAGIVSPVK and 5′-TTIA(A/G)(C/T)TC(A/G)TC(C/T)TCIGCICC-3′ corresponding to the antisense orientation of GAEDELK; and of two peptides from P17: 5′-GGIGTIGTIGA(A/G)AA(C/T)GCIAT(C/T)AA-3′ corresponding to the sense orientation of GVVENAIN and 5′-TTIA(A/G)(A/G)TC(C/T)TG(A/G)TA(A/G)TA-IA-3′ corresponding to the antisense orientation of VYYQDLN. These oligonucleotides were used as primers to amplify cDNA specific to the two proteins by PCR. Total RNA isolated from calli grown for 28 d in the presence of 2.5 μM 2iP, i.e. nonregenerating calli, was used as a template for single-stranded cDNA synthesis (BRL Superscript Preamplification System). Five microliters of single-stranded cDNA were diluted to 100 μL in 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mg mL^{-1} BSA, 0.2 mM each of dATP, dCTP, dTTP, and dGTP, 1.3 μM each of sense and antisense oligonucleotides, and 2 to 5 units of *Taq* polymerase. Five cycles of 30-s denaturation at 94°C, 2-min annealing at 50°C, and 30-s extension at 72°C (+2 s per cycle) were followed by 30 cycles of amplification in the same conditions except for a lower annealing temperature of 40°C. Amplified cDNA with the expected size (350 bp and 620 bp for P21 and P17, respectively) were purified by agarose gel electrophoresis and cloned in a Bluescript II KS(+)-derived T-vector (Marchuk et al., 1990).

The cloned PCR products were used to screen about 75,000 plaques of a *Petunia* flower cDNA library in λGEM 4 vector (Benton and Davies, 1977). Hybridization with probes ^{32}P-labeled by random-priming (Quick Prime Kit, Pharmacia) was performed for 15 h at 60°C according to Church and Gilbert (1984). After subcloning, the DNA of positive plaques was isolated by a minipreparation procedure (Kao et al., 1982), and the

cDNA were rescued as pGEM1 derivatives by *Spe*I excision from the DNA phages as recommended by the manufacturer (Promega). The excised phagemids were purified on gels and recircularized by ligation. The dideoxy method (Sanger et al., 1977; Sambrook et al., 1989) was used for sequencing double-stranded DNA.

RESULTS

Microsequencing Led to the Identification of Two Proteins Induced by Lowering Cytokinin

P21 and P17 belong to a group of four proteins that accumulate in *Petunia* calli under nonregeneration conditions because of a too-low concentration of cytokinin in the medium (Renaudin et al., 1991). They were purified by 2D-PAGE and partly hydrolyzed into peptides by trypsin. N-terminal sequences of several peptides were obtained for each of the two proteins. Three out of the five peptides from P21 (Fig. 1) were the most homologous to aleurain and oryzain γ, thiol proteases from barley and rice, respectively (Rogers et al., 1985; Watanabe et al., 1991). The four peptides from P17 (Fig. 2) were highly homologous to anionic peroxidases from potato (Roberts et al., 1988) and tomato (Roberts and Kolattukudy, 1989).

Cloning of PeTh3, a *Petunia* cDNA for a Thiol Protease

Three peptides from P21 could be aligned with thiol proteases, so we prepared degenerate oligonucleotides corresponding to two of these peptides to amplify homologous cDNA by PCR. A PCR fragment of 350 bp was amplified and cloned. The predicted sequence was similar to thiol proteases in data banks (data not shown). This cDNA was subsequently used as a probe for the screening of a cDNA library from young *Petunia* flowers. Five clones of different lengths, from 1.0 to 1.4 kbp, were isolated from the library. The overlapping sequences were more than 98% identical, suggesting that they all represented the same mRNA. The longest cDNA was called PeTh3. Its complete nucleotide and deduced amino acid sequences are shown in Figure 1. Its open reading frame, comprising 1077 nucleotides, is likely to be complete, since the sequence around the potential initiation codon is similar to ATG initiation consensus sequences in plants (Lütcke et al., 1987). All five peptide sequences from P21 matched almost perfectly with the sequence translated from PeTh3, which favors the fact that this cDNA actually encodes P21. The only mismatch was between T^{144} in the sequence translated from PeTh3 (Fig. 1) and an F in the peptide sequence from P21. The T^{144} is highly conserved between plant thiol proteases (Fig. 3), which suggests a protein sequencing error. Southern blot experiments showed that the PeTh3 sequence was likely to be present as a single gene in the *Petunia* genome (data not shown).

The protein encoded by PeTh3 is very similar to three thiol proteases, barley aleurain, rice oryzain γ, and mammalian cathepsin H (Table I). It is also very similar to SAG2, a senescence-associated thiol protease of Arabidopsis (Hensel et al., 1993), of which only a partial sequence of

```
                                M  S  R  L  S  L  L  L  V  L  V  A  12
        GTT GCT GTC GTT ACA ATG TCT CGT CTG TCA CTC CTA TTG GTT CTA GTC GCC  51

G  L  F  A  V  A  F  A  R  T  A  N  F  A  D  E  N  P  I  31
GGT CTT TTC GCC GTC GCA TTC GCC CGA ACG GCG AAC TTC GCC GAT GAG AAT CCG ATC 108

R  Q  V  V  S  D  S  F  H  E  L  E  S  G  I  L  H  V  V  50
AGG CAA GTT GTT TCG GAC AGT TTC CAT GAA CTG GAA AGC GGA ATT CTC CAT GTG GTC 165

G  Q  T  R  H  A  L  S  F  A  R  F  A  R  R  Y  G  K  R  69
GGC CAG ACT CGT CAT GCT CTC TCC TTC GCT CGC TTT GCT CGC AGG TAT GGG AAG AGG 222

Y  D  S  V  E  E  I  K  Q  R  F  D  I  F  L  D  N  L  E  88
TAC GAT TCA GTT GAG GAG ATC AAG CAA AGG TTC GAC ATA TTT TTG GAC AAT TTG GAG 279

M  I  N  S  H  N  D  K  G  L  S  Y  K  L  G  V  N  E  F  107
ATG ATT AAT TCG CAT AAT GAC AAA GGA CTG TCA TAC AAA CTC GGT GTC AAT GAG TTT 336

S  D  L  T  W  D  E  F  R  R  D  R  L  G  A  A  Q  N  C  126
AGT GAC CTA ACA TGG GAT GAA TTT CGG AGA GAT AGG CTG GGG GCA GCT CAA AAC TGT 393

S  A  T  T  K  G  N  L  K  L  R  D  A  V  L  P  E  T  K  145
TCT GCT ACA ACA AAG GGC AAT CTC AAG CTC CGT GAC GCT GTT TTG CCA GAG ACG AAA 450

D  W  R  E  A  G  I  V  S  P  V  K  N  Q  G  K  C  G  S  164
GAC TGG AGG GAA GCT GGG ATT GTC AGC CCA GTC AAG AAT CAG GGC AAG TGC GGT TCT 507

C  W  T  F  S  T  T  G  A  L  E  A  A  Y  T  Q  K  F  G  182
TGC TGG ACA TTC AGC ACC ACT GGT GCA TTG GAG GCA GCA TAT ACC CAA AAA TTT GGG 564

K  G  I  S  L  S  E  Q  Q  L  V  D  C  A  G  A  F  N  N  202
AAG GGA ATC TCG CTA TCT GAG CAG CAG CTT GTA GAC TGT GCT GGA GCT TTT AAT AAC 621

F  G  C  N  G  G  L  P  S  Q  A  F  E  Y  I  K  S  N  G  221
TTT GGC TGC AAC GGT GGA CTC CCA TCA CAA GCC TTT GAG TAC ATT AAA TCC AAT GGT 678

G  L  E  T  E  E  A  Y  P  Y  T  G  K  N  G  L  C  K  F  240
GGT CTT GAA ACT GAA GAA GCA TAT CCA TAC ACC GGC AAG AAC GGC TTA TGT AAA TTC 735

S  S  Q  N  V  G  V  K  V  T  D  S  V  N  I  T  L  G  A  259
TCA TCA CAA AAT GTT GGT GTC AAA GTC ACT GAT TCT GTC AAT ATT ACC TTG GGT GCT 792

E  D  E  L  K  Y  A  V  A  L  V  R  P  V  S  V  A  F  E  278
GAA GAT GAA TTA AAA TAC GCT GTT GCT CTG GTT AGG CCC GTT AGT GTT GCT TTT GAG 849

V  V  K  G  F  K  Q  Y  K  S  G  V  Y  T  S  T  E  C  G  297
GTA GTT AAA GGT TTC AAA CAG TAC AAG AGC GGA GTT TAT ACC AGC ACC GAG TGC GGA 906

T  T  P  M  D  V  N  H  A  V  L  A  V  G  Y  G  V  E  Y  316
ACC ACT CCC ATG GAT GTA AAC CAT GCT GTT CTT GCT GTG GGT TAC GGT GTT GAA TAT 963

G  V  P  F  W  L  I  K  N  S  W  G  A  D  W  G  D  N  A  335
GGT GTT CCC TTT TGG CTC ATC AAG AAC TCA TGG GGA GCA GAT TGG GGT GAC AAT GCA 1020

Y  F  K  M  E  M  G  N  D  M  C  G  I  A  T  C  A  S  Y  354
TAT TTC AAA ATG GAG ATG GGT AAC GAC ATG TGT GGT ATT GCA ACT TGC GCA TCC TAC 1077

P  V  V  A                                                  358
CCT GTC GTT GCT TGA GGT TCG GAG TAT GTA AAA TTT TTT CAC CAA AGG GAA GTA GAC 1134

ATA AAC CCC ACG TAG TCC TAG TTG ATG ATG CGG AAA ACA TCC AAG CTC CAT ACT CAG 1191

ATA CTT GTA TTA ATT GGA GAT CTC TAT GCA CGC GAA GAT GGT TTA GGC TAC TTA GTG 1248

AAA GAA TAA GAG ACA GGA AGC TGA ATT CAC CAG ACA TAA ATT ATG AGA ATA ATC CCT 1305

ATG TAA ACT ATA CTT GTG TAA GTC ATG GGT TTA AAA AAA GGA AAA AAA AAA AAA     1359
```

Figure 1. Nucleotide and deduced amino acid sequence of PeTh3 cDNA. Tryptic peptide sequences obtained from P21 are underlined. They are identical to the deduced amino acid sequence of PeTh3, except for an F instead of T^{144} in the sequence translated from PeTh3.

95 residues is known (Table I; Fig. 3). The similarity of PeTh3 to other plant thiol proteases, notably from Solanaceae such as tobacco and tomato, is less (about 38% identity, 50% similarity, data not shown). The alignment of PeTh3 with thiol proteases from various origins shows that PeTh3 has the structural features of typical thiol proteases (Fig. 3). The amino acids C^{165} and H^{305} in PeTh3 are conserved among thiol proteases, and they have been implicated in the catalytic reaction (Baker, 1980). Similarly, Q^{159}, N^{305}, S^{326}, and W^{327}, implicated in the structure of the active site of thiol proteases (Baker, 1980), are conserved in PeTh3. Finally, PeTh3 has six conserved Cys residues at positions 162, 196, 205, 238, 297, and 346 that are involved in disulfide bridge formation and that are typical of thiol proteases.

The molecular mass of P21, estimated from 2D gels (28 kD), is much less than that of the protein predicted from

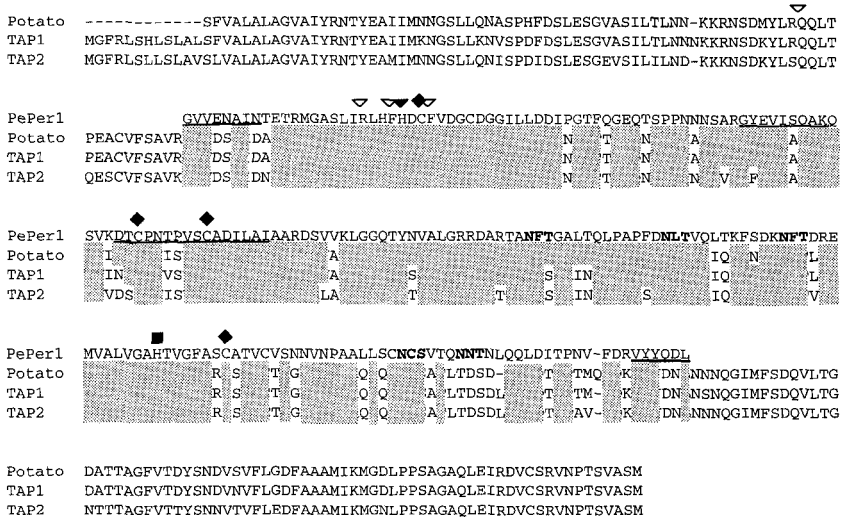

Figure 2. Comparison of the deduced amino acid sequence of PePer1 with anionic peroxidases from potato (Roberts et al., 1988) and tomato TAP1 and TAP2 (Roberts and Kolattukudy, 1989). Tryptic peptide sequences obtained from P17 are underlined in the PePer1 sequence. The multisequence alignment was made using the program MULTALIN (Corpet, 1989). Shaded areas show sequences identical to PePer1. Dashes indicate gaps required for optimal alignment. Conserved Cys residues implicated in intramolecular disulfide bridge linkage are indicated by a diamond. Conserved histidines involved in acid/base catalysis and in the binding with heme iron are indicated by a solid triangle and square, respectively. Amino acids forming the catalytic site are indicated by open triangles. Amino acids in bold show potential glycosylation sites in PePer1.

PeTh3 (39.2 kD). A similar situation also occurs in the case of aleurain. It is known that aleurain is first synthesized as a propeptide, of which the N-terminal 140-residue stretch is further cleaved (Holwerda et al., 1990). The PeTh3 N-terminal sequence beginning with a charged amino acid (R^3) followed by 17 hydrophobic residues is typical of a signal sequence (Chrispeels, 1991). There is also a partial homology between F^{24} and R^{31} in PeTh3 with two vacuolar targeting determinants that have been characterized in aleurain (Holwerda et al., 1992; Holwerda and Rogers, 1993) (Fig. 3). P21, aleurain, and oryzain γ are also highly similar to human cathepsin H, but the homology is less in the N-terminal domain, which is a further indication of the specific targeting function of this domain.

After removal of its N-terminal targeting signal, the cathepsin H protein has been reported to give rise, by proteolytic cleavage, to a peptide minichain, EPQNCSAT, which is bound by a disulfide bridge to a Cys in the C terminus of the enzyme (Ritonja et al., 1988). A similar structural organization was also reported in aleurain (Holwerda et al., 1992, Holwerda, 1993). It is also conserved in oryzain γ and PeTh3 (Fig. 3): the octapeptide stretch A^{122}AQNCSAT in PeTh3, is almost identical to the human cathepsin H minichain, and there is a conserved Cys residue in the C-terminal part of the four thiol proteases (C^{351}in PeTh3) that is involved, in cathepsin H, in binding to the minichain. This structural organization of the minichain region and at the C terminus is not conserved in all thiol proteases, but it is typical of these four proteins, as is the presence of two conserved potential glycosylation sites at positions N^{125} and N^{254} in PeTh3 (Fig. 3).

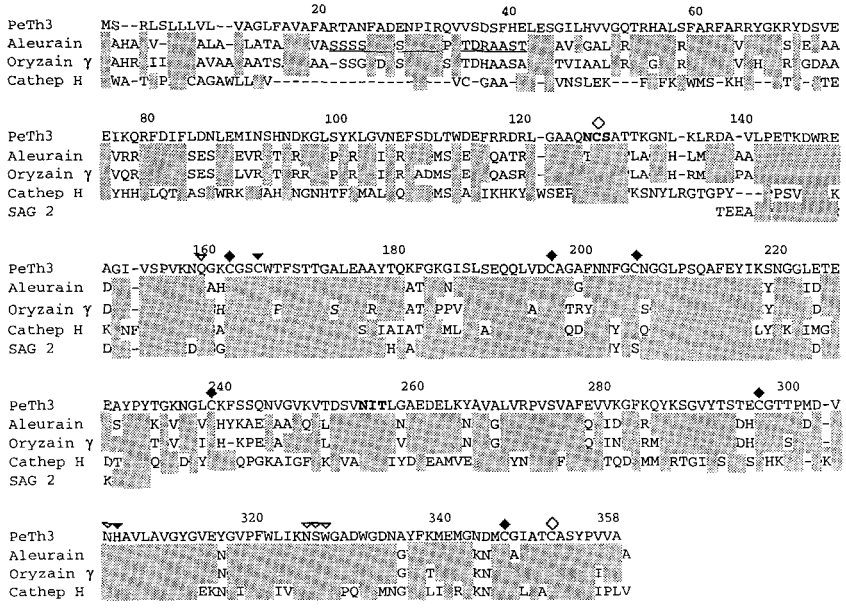

Figure 3. Comparison of the deduced PeTh3 amino acid sequence with the most homologous thiol proteases: barley aleurain (Rogers et al., 1985), rice oryzain γ (Watanabe et al., 1991), human cathepsin H (Fuchs et al., 1988), and Arabidopsis SAG2 (Hensel et al., 1993). The multisequence alignment was made using the program MULTALIN (Corpet, 1989). Shaded areas show sequences identical to PeTh3. Dashes indicate gaps required for optimal alignment. Solid diamonds show conserved cysteines predicted to form intrachain disulfide bonds in all thiol proteases; open diamonds show cysteines implicated, for cathepsin H, in binding the octapeptide minichain EPQNCSAT to the C terminus of the protein. Amino acids involved in active site formation and in the catalytic reaction are indicated by open and solid triangles, respectively. The vacuolar targeting determinants of aleurain are underlined. Amino acids in bold show potential glycosylation sites in PeTh3.

Table I. *Homology of P21, a thiol protease from Petunia, with other thiol proteases from plants and animals*

The sequences have been aligned with the program MULTALIN (Corpet, 1989). Data are expressed as percent identity with P21 and, in parentheses, as percent similarity. Aleurain is from barley (Rogers et al., 1985), oryzain γ from rice (Watanabe et al., 1991), and cathepsin H from human (Fuchs et al., 1988). The Arabidopsis SAG2 sequence (Hensel et al., 1993) does not include the complete open reading frame of the protein but only 95 residues (Fig. 3). –, Incomplete sequence.

Thiol Protease	Whole Polypeptide Sequence	SAG2 Overlapping Sequence
Aleurain	71 (76)	86 (86)
Oryzain γ	67 (73)	79 (83)
SAG2	–	86 (91)
Cathepsin H	46 (51)	65 (68)

Cloning of PePer1, a *Petunia* cDNA for an Anionic Peroxidase

A strategy similar to that described above was followed to search for a P17-specific cDNA. Two oligonucleotides deduced from peptide sequences of P17 enabled the amplification by PCR and the cloning of PePer1, a 615-bp cDNA. This size was expected from the alignment of P17 peptide sequences with peroxidases. The cDNA PePer1 encodes a 205-amino acid sequence in which the four tryptic peptides identified by peptide sequencing from P17 are present (Fig. 2).

Attempts to recover a complete cDNA homologous to PePer1 in the *Petunia* cDNA library from flowers were unsuccessful. Nevertheless, the length of PePer1 was sufficient for a structural analysis. The deduced protein sequence of PePer1 was most homologous (77–80% identity, 84–86% similarity) to anionic peroxidases of potato (Roberts et al., 1988) and tomato (Roberts and Kolattukudy, 1989). Further homologies, e.g. with other anionic peroxidases, were below 60% (data not shown). The molecular mass of P17 as estimated from 2D gels, 50 kD, was the same as that of the proteins deduced from the potato and tomato peroxidase cDNA. Amino acids that have been implicated in the catalytic reaction and in the binding of iron with the heme prosthetic group are all present in the PePer1-deduced amino acid sequence (Fig. 2), which strongly reinforces the identification of P17 as a bona fide peroxidase. Southern blot experiments revealed the presence in *Petunia* of at least four fragments of genomic DNA able to hybridize to PePer1, of which three provided strong hybridization signals, which indicates a small gene family for this enzyme in *Petunia* (data not shown).

Expression of P21 and P17 Proteins in *Petunia* calli

To approach the effect of cytokinin independently of organogenesis, we analyzed the expression of P21 and P17 in *Petunia* line St40, which is unable to regenerate in the conditions described above for the PC6 line. Although protoplasts and calli could repeatedly be obtained from the line St40 with a good efficiency, only unorganized callus growth occurred, rather than regeneration, whatever the concentrations of auxin and cytokinin in medium B (Fig. 4, A and B, and data not shown). It is important to note that the growth of St40 calli was dependent on cytokinin, since calli grown in the presence of 2.5 μM 2iP were less than 50% of the size of those grown in the presence of 25 μM 2iP (Fig. 4A).

Calli from the lines PC6 and St40 were cultured on medium B in the presence of 2.5 or 25 μM 2iP, and the abundances of proteins P21 and P17 were checked in the calli after 28 or 36 d of culture. The 2D-PAGE patterns from

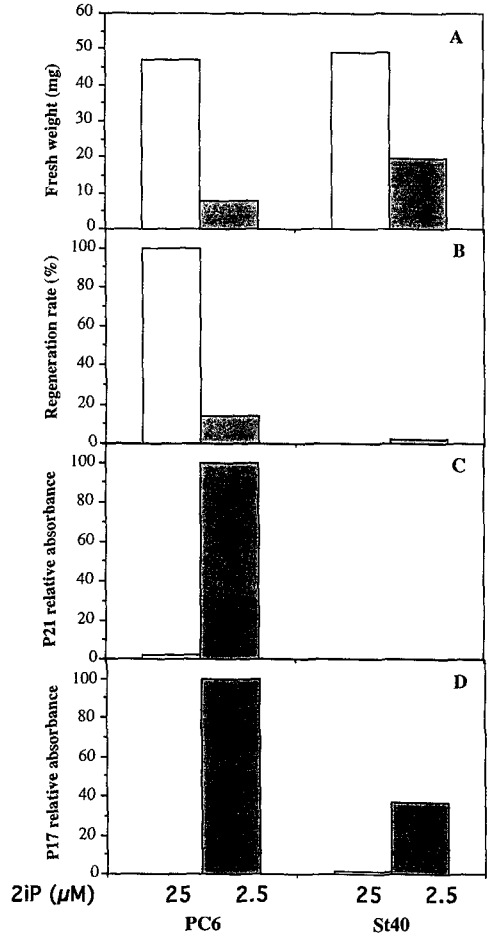

Figure 4. Growth, regeneration, and accumulation of P21 and P17 proteins in calli from the *Petunia* lines PC6 and St40 as a function of cytokinin concentration. Protoplast-derived calli grown for 21 d in medium C were further cultured on medium B containing either 2.5 or 25 μM 2iP. The culture period was 28 d for PC6 and 36 d for St40. The data are from one typical experiment. A, Mean fresh weight of the calli at the end of the culture period. B, Regeneration rate measured as the relative number of calli forming buds at the end of the culture period, over a total of 36 to 48 calli. C and D, Absorbance of P21 (C) and P17 (D) proteins as shown by densitometric measurement of P21 and P17 spots on 2D gels. The absorbance of P21 and P17 is expressed relative to the absorbance of 12 spots remaining at constant level in all conditions and is further normalized as percent of the maximum value (Renaudin et al., 1991). The data represent the mean value of absorbances on three 2D gels, with the SD values being below 15%.

the lines PC6 and St40 were mostly similar (Fig. 5). P21 and P17 were putatively identified in 2D-PAGE patterns from St40 as spots located in exactly the same place as in PC6 patterns. P21 and P17 were the most highly expressed in PC6 calli when they were cultured in the presence of 2.5 μM 2iP, i.e. in nonregenerating conditions (Fig. 4, C and D). However this was not the case for line St40, the calli of which did not regenerate on the cytokinin-rich medium. P21 was present at a negligible level in St40 calli whatever the cytokinin concentration. P17 was detected in St40, as in PC6, only in the presence of the lowest concentration of cytokinin, although its abundance was less than 50% of that from PC6 (Fig. 4D).

Expression of P21 and P17 mRNA in *Petunia* calli

The abundance of P21 and P17 mRNA in St40 and PC6 calli was estimated by northern blotting. A 1.4-kb transcript was detected with a P21-specific probe (Fig. 6). In PC6, the level of P21 mRNA was higher after 28 d than after 7 d. It was also consistently higher in the presence of the lower cytokinin concentration. The differential effect of cytokinin concentration on P21 mRNA level occurred as soon as d 7, i.e. 7 d earlier than the effect on P21 protein (Renaudin et al., 1991), and it was more pronounced at that time than at d 28. The abundance of P21 mRNA in St40 calli was less than in PC6 calli. It was barely detectable at d 7 and accumulated to a significantly higher level, independently of cytokinin concentration, at d 28.

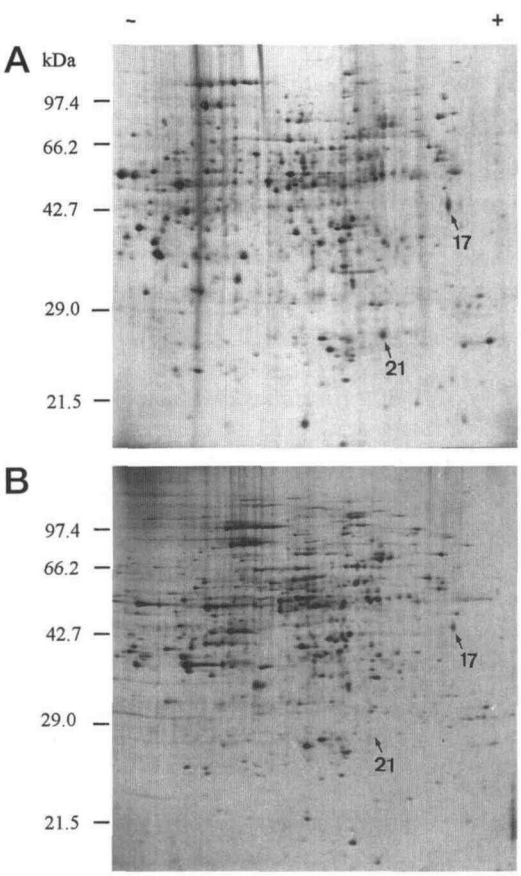

Figure 5. 2D protein patterns of PC6 and St40 calli. Protoplast-derived calli grown for 21 d in medium C were further cultured onto medium B containing 2.5 μM 2iP for 28 d in the case of PC6 (A) and for 36 d in the case of St40 (B). Fifty micrograms of proteins were separated by 2D-PAGE and silver stained. The locations of P17 and P21 are indicated by arrows.

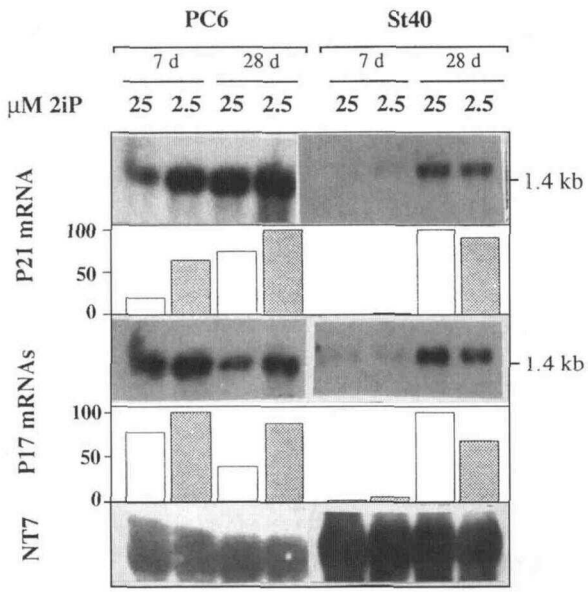

Figure 6. Northern blot analysis of PeTh3 and PePer1 expression in calli from the *Petunia* lines PC6 and St40. Total RNA were extracted after 7 and 28 d culture of calli on medium B in the presence of 2.5 μM or 25 μM 2iP. They were successively hybridized with PCR-amplified cDNA corresponding to P21 (a 350-bp cDNA also used for the cloning of PeTh3) and to P17 (PePer1, a 615-bp cDNA) and with NT7, a tobacco 25 S rRNA. The histogram below each photograph panel shows the result of densitometric measurement of the autoradiographs. For each line, the absorbances were normalized with the signals of ribosomal RNA and are expressed as percent of the maximum value.

PePer1 hybridized with 1.4-kb RNA (Fig. 6), which will be further referred to as P17 mRNA, although the possibility that several mRNA species hybridize with Peper1 cannot be excluded from Southern blot data. The pattern of accumulation of P17 mRNA was very different in the two lines PC6 and St40. P17 mRNA were present in PC6 to a slightly higher level at d 7 than at d 28, whereas in St40 calli, P17 mRNA were present at a much lower level at d 7 than at d 28. Moreover, the level of P17 mRNA varied differently in the two lines as a function of cytokinin concentration: in PC6 calli, it was higher in the presence of 2.5 μM 2iP than in the presence of 25 μM 2iP, whereas the opposite situation occurred in 28-d-old St40 calli.

Expression of P21 and P17 mRNA in *Petunia* plants

We performed northern blot analysis with RNA from leaves, roots, and flowers at different developmental stages (Fig. 7). For lines PC6 and St40, the level of P21 and P17 mRNA varied according to the type of organ and the developmental stage. However, the pattern of expression was otherwise significantly different between PC6 and

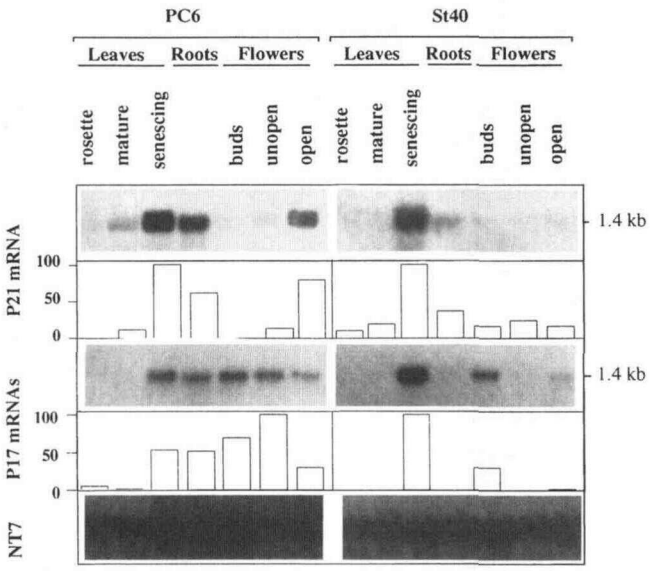

Figure 7. Northern blot analysis of PeTh3 and PePer1 expression in organs from *Petunia* plants of the lines PC6 and St40. Total RNA were extracted from rosette, mature and senescing (yellow) leaves, roots and flower buds, young unopened flowers, and mature flowers. They were successively hybridized and quantitatively analyzed, as described in Figure 6.

St40. P21 mRNA was almost undetectable in young rosette leaves and flower buds from the line PC6. It became more abundant in leaves and in flowers during aging, and it was also relatively abundant in roots. In line St40, P21 mRNA was almost undetectable in young and mature leaves and in flowers whatever their age, but it accumulated to high levels in senescing leaves and, to a lesser extent, in roots. In both lines, the highest level of P21 mRNA expression was encountered in senescing leaves.

P17 mRNA were almost undetectable in young and mature leaves from line PC6 (Fig. 7). They were highly accumulated, in this line, in old leaves, roots, and unopen flowers. In St40, P17 mRNA were only detected in senescing leaves and flower buds.

DISCUSSION

We have previously analyzed the cytokinin-controlled transition from callus to vegetative bud by describing the 2D-protein patterns of *Petunia* calli during this process (Renaudin et al., 1991). The abundance of a group of proteins increased after subculture of the calli in the presence of a concentration of cytokinin too low to achieve organogenesis. They attracted our interest because their induction occurred earlier than macroscopic differences, e.g. size, color, and regeneration, in calli cultured in the presence of a low, as compared with a high, concentration of cytokinin. In the present study, we show that two of these proteins, P21 and P17, are a thiol protease and an anionic peroxidase, respectively. We have cloned two corresponding cDNA, PeTh3 and PePer1, and we present data about the mechanisms that trigger their expression in vitro.

Structural Analysis and Expression of the Thiol Protease

Several criteria indicate that PeTh3 is the cDNA actually encoding P21. PeTh3 contains all the sequences of tryptic peptides of P21, and it encodes a protein of the same molecular weight as P21. Finally, it occurs as a single gene in the *Petunia* genome. PeTh3 has a complete open reading frame which, when translated, has the constitutive features of a thiol protease. Thiol (Cys) proteases (EC 3.4.22), are a group of proteases with related structures that contain an essential Cys residue involved in a covalent intermediate complex with the substrate (Bond and Butler, 1987). Thiol proteases are assigned various functions within animal and plant cells, among which is the extensive degradation of proteins during their routine turnover in the cytosol, lysosomes, or vacuoles (Bond and Butler, 1987). In plants, the most extensively characterized thiol proteases are connected with the mobilization of seed reserves (Holwerda and Rogers, 1993). PeTh3 is highly homologous to two such thiol proteases from barley, aleurain (Rogers et al., 1985), and rice, oryzain γ (Watanabe et al., 1991), which are specifically induced in aleurone layers during germination. Recently, a partial cDNA named SAG2, coding for a thiol protease closely related to aleurain and oryzain γ, was cloned in Arabidopsis leaves where it was induced early during senescence (Hensel et al., 1993).

Sequence analysis shows that aleurain, oryzain γ, P21, the protein encoded by SAG2, and mammalian cathepsin H form a highly conserved subgroup of thiol proteases (Table I; data not shown). Remarkably, cathepsin H and aleurain are the only two proteases that have been shown so far to possess an aminopeptidase activity in addition to endoprotease activity (Schwartz and Barrett, 1980; Holwerda and Rogers, 1993). Both of them belong to the same structural subgroup, so the high level of conservation among this subgroup is likely related to this functional similarity.

RNA and protein analyses showed that PeTh3 was differentially expressed according to developmental and environmental conditions. The large variation of PeTh3 mRNA indicated the regulation of expression at the transcriptional level. However, PeTh3 mRNA were found in two situations in which no P21 protein was detected: in 7-d-old calli from the line PC6 (Renaudin et al., 1991; Fig. 6) and in 28-d-old calli from the line St40 (Figs. 4C, 6). Thus, although our northern blotting protocol may be more sensitive than densitometric measurement of the protein on 2D gels, posttranscriptional regulation of PeTh3 expression cannot be excluded.

PeTh3 mRNA was found in all of the organs of *Petunia* plants. Its abundance increased in leaves and flowers during aging and culminated in senescing leaves. This pattern of expression, together with the structural similarity, makes PeTh3 the *Petunia* homolog of Arabidopsis SAG2 (Hensel et al., 1993). In the absence of data relating their expression to senescence, this comparison cannot be extended to the other plant thiol proteases of the same group. It should be stressed, however, that, whereas oryzain γ is expressed only in germinating seeds and not in shoots or roots (Watanabe et al., 1991), the expression of aleurain was not restricted to aleurone cells, but rather it also occurred

constitutively at high levels in leaf and root tissues (Rogers et al., 1985).

Structural Analysis and Expression of the Anionic Peroxidase

A truncated cDNA, PePer1, was cloned by using peptide sequences from P17. The identification of P17 as an anionic peroxidase is very likely because PePer1 has all of the peptides sequenced from P17, and P17 has the same molecular weight as peroxidases homologous to PePer1. However, whether PePer1 is actually the cDNA specific to P17 remains unknown. Southern blot hybridization revealed the presence of approximately four homologous genes in the *Petunia* genome. Thus, the assay of mRNA by northern blotting using PePer1 as probe provides only a preliminary analysis of the combined expression of all of these genes. Peroxidases are considered reliable indicators of senescence in plant tissues (Birecka et al., 1979; Gaspar et al., 1982; Abeles et al., 1988; Tadeo and Primo-Millo, 1990). It was thus not surprising to find a high expression of P17 mRNA in senescing *Petunia* leaves. But, in contrast to the case of P21 mRNA, P17 mRNA were also more abundant in young flower buds than in open mature flowers. The expression of p17 mRNA in plants was thus not correlated with aging in all organs.

The abundance of P17 did not parallel that of the mRNA hybridizing to PePer1 in all cases. These latter were more abundant in 7-d-old PC6 calli than in 28-d-old calli, whereas P17 was not detected in the former (Renaudin et al., 1991, Figs. 5D, 7). In 28-d-old St40 calli, mRNA hybridizing to PePer1 were slightly more abundant in the presence of the higher cytokinin concentration, although there was significantly less P17 protein. These results are likely to be due to the occurrence of several P17-related mRNAs, although the posttranscriptional control of P17 abundance, which is especially relevant in the case of peroxidases, cannot be excluded. The diversity of peroxidase isoforms in plants arises by various posttranslational modifications of a smaller number of gene products (Gaspar et al., 1982). In *Petunia*, three structural genes encode the three major peroxidase isoenzyme families in leaf extracts, two of which are anionic (Van der Berg and Wijsman, 1981). Whether P17 belongs to one of these families remains an open question.

PePer1 has a high homology with two *Solanum* anionic peroxidases, one from potato and two, TAP1 and TAP2, from tomato. As with PeTh3, this strikingly high homology likely reflects some common functional properties, although the functions performed in vivo by these enzymes remain unknown. The potato and tomato peroxidases have not, as yet, been associated with senescence. They are induced in tomato fruits and potato tubers by perturbations leading to suberization, such as pathogen infection, elicitor or ABA treatments, or wound healing (Roberts et al., 1988; Roberts and Kolattukudy, 1989). ABA also induces their expression in potato and tomato callus (Roberts and Kolattukudy, 1989). However, the expression of TAP1 is also developmentally regulated in the exocarp of green tomato fruits (Sherf and Kolakuttudy, 1993), which suggests that the peroxidase could fulfill an alternative function in the plant other than that related to defense response mechanisms.

P21 and P17 Are Early Markers of a Senescence Program Triggered by a Low Concentration of Cytokinin

The induction of PeTh3 and PePer1 in *Petunia* calli from line PC 6 was detected after 7 d (mRNA) or 14 d (proteins) (Renaudin et al., 1991) of subculture in the presence of a low, as compared with a high, concentration of cytokinin. At these times, callus growth and morphology were the same in the presence of either concentration of cytokinin. Calli cultured on the low cytokinin medium stopped growing after 3 weeks (Fig. 4A) (Renaudin et al., 1991) and became senescent about 2 weeks later. Considering that senescing leaves display a high level of expression of PeTh3 and PePer1, the induction of these genes in calli may be considered to be associated with a phenomenon triggered by the low level of cytokinins, i.e. the onset of senescence. Several genes have already been shown to be down-regulated by cytokinins, but their association with a biological response controlled by cytokinins remains unclear (Crowell and Amasino, 1991; Watillon et al., 1991; Binns, 1994; Teramoto et al., 1994). It is interesting to note that SAM22, a gene induced by cytokinin starvation in soybean cells (Crowell and Amasino, 1991), was also the most expressed in senescing leaves, in addition to be developmentally and stress regulated (Crowell et al., 1992).

Although it is likely that all of the phytohormones are involved in the control of senescence, most research has concentrated on the role of cytokinins (Smart, 1994). When applied exogenously, cytokinins exert a senescence-retarding activity on leaf tissues (Noodén and Letham, 1993; Smart, 1994), and recent results from the assay of endogenous cytokinins (Singh et al., 1992; Noodén and Letham, 1993) and overproduction of cytokinin in transgenic plants (Smart et al., 1991; Li et al., 1992) have confirmed that cytokinins are one of the endogenous signals regulating leaf senescence. Our results suggest that lowering the total cytokinin content of calli, which ultimately results in growth arrest and senescence, induces the expression of PeTh3 and PePer1 at an early stage of this process. PeTh3 and PePer1 are likely to be two of the many genes whose expression is modified by senescence, since this phase of plant development is accompanied by major changes in gene expression (Becker and Apel, 1993; Hensel et al., 1993; Lohman et al., 1994; Smart, 1994). The up-regulation of a thiol protease activity in this context, as also demonstrated in senescing Arabidopsis (Hensel et al., 1993; Lohman et al., 1994) and maize (Smart et al., 1995) leaves, would be in the initiation of nutrient mobilization during the early stages of senescence, as well as in the autolytic process in later stages.

The expression pattern of PeTh3 and PePer1 was found to be somewhat different in line St40 compared with line PC6. In whole plants, these genes were mostly expressed only in senescing leaves of St40, whereas in PC6 they were also expressed in younger leaves and in other organs, although at a level somewhat lower than that found in senescing leaves. In calli, the expression of PeTh3 and

PePer1 at the RNA level was much greater in older calli (after 28 d of culture) than in younger calli (after 7 d of culture) in St40, whereas for PC6, the difference of expression between these two growth periods was not as great. These data suggest that the expression of PeTh3 and PePer1 is more tightly associated with late stages of senescence in St40 than in PC6.

Leaf explants of the line St40 regenerate rapidly and with a high efficiency (Auer et al., 1992), higher than the line PC6 (Dulieu et al., 1983). Surprisingly, protoplast-derived St40 calli turned out to be unable to regenerate buds with the protocol described for PC6. Moreover, St40 calli did not regenerate whatever the hormonal content (auxin and cytokinin) in the callus growth medium and during the regeneration step. When growth occurred, it led to actively proliferating, unorganized calli (data not shown). The opposite results of the two *Petunia* lines for regenerating from leaf explants or from protoplast-derived calli further demonstrate the strong interaction between the differentiation status of plant explants and their competence toward regeneration (Halperin, 1988). The high efficiency of regeneration from leaf explants of the St40 line has been associated with its high rate of uptake and metabolism of BA, an exogenous cytokinin (Auer et al., 1992), and its capacity to form a BA disaccharide conjugate (Auer and Cohen, 1993), which suggests that a functional difference with the line PC6, considering regeneration, is related to cytokinin signaling.

The two *Petunia* lines PC6 and St40 respond differently to in vitro organogenesis and senescence-associated gene expression. Cytokinins are a common signal for these otherwise different developmental pathways. Although the genetic backgrounds of the two lines are quite different, our results suggest that they are likely to display a major difference in cytokinin metabolism and/or sensitivity, which accounts for differences in two cytokinin-controlled responses: regeneration and senescence.

ACKNOWLEDGMENTS

We thank A. Gerats for the gift of the *Petunia* flower cDNA library and D.C. Logan for careful reading of the manuscript.

Received October 24, 1995; accepted January 30, 1996.
Copyright Clearance Center: 0032-0889/96/111/0159/10.
The accession numbers for the sequences reported in this article are U31094 (PeTh3) and U31095 (PePer1).

LITERATURE CITED

Abeles FB, Dunn LJ, Morgens P, Callahan A, Dinterman RE, Schmidt J (1988) Induction of 33-kD and 60-kD peroxidases during ethylene-induced senescence of cucumber cotyledons. Plant Physiol **87:** 609–615

Auer CA, Cohen JD (1993) Identification of a benzyladenine disaccharide conjugate produced during shoot organogenesis in *Petunia* leaf explants. Plant Physiol **102:** 541–545

Auer CA, Cohen JD, Laloue M, Cooke TJ (1992) Comparison of benzyladenine metabolism in two *Petunia hybrida* lines differing in shoot organogenesis. Plant Physiol **98:** 1035–1041

Baker EN (1980) Structure of actinidin after refinement at 1.7 Å resolution. J Mol Biol **141:** 441–484

Bauw G, De Loose M, Inzé D, Van Montagu M, Vandekerckhove J (1987) Alterations in the phenotype of plant cells studied by NH2-terminal amino acid sequence-analysis of proteins electroblotted from two dimensional gel-separated total extracts. Proc Natl Acad Sci USA **84:** 4806–4810

Becker W, Apel K (1993) Differences in gene expression between natural and artificially induced leaf senescence. Planta **189:** 74–79

Benton WD, Davies RW (1977) Screening λgt recombinant clones by hybridization to single plaques *in situ*. Science **196:** 180–182

Binns AN (1994) Cytokinin accumulation and action: biochemical, genetic, and molecular approaches. Annu Rev Plant Physiol Plant Mol Biol **45:** 173–196

Birecka H, Chaskes MJ, Goldstein J (1979) Peroxidases and senescence. J Exp Bot **30:** 565–573

Bond JS, Butler PE (1987) Intracellular proteases. Annu Rev Biochem **56:** 333–364

Chrispeels MJ (1991) Sorting of proteins in the secretory system. Annu Rev Plant Physiol Plant Mol Biol **42:** 21–53

Church GM, Gilbert W (1984) Genomic sequencing. Proc Natl Acad Sci USA **81:** 1991–1995

Corpet F (1989) Multiple alignment with hierarchical clustering. Nucl Acids Res **16:** 10881–10890

Crowell DN, Amasino RM (1991) Induction of specific mRNAs in cultured soybean cells during cytokinin or auxin starvation. Plant Physiol **95:** 711–715

Crowell DN, John ME, Russell D, Amasino RM (1992) Characterization of a stress-induced, developmentally regulated gene family from soybean. Plant Mol Biol **18:** 459–466

Davies RW, Thomas M, Cameron J, St John TP, Scherer S, Padgett S (1980) Rapid DNA isolation for enzymatic and hybridization analysis. Methods Enzymol **65:** 404–411

Dulieu HL, Bruneau R, Pelletier A (1983) Heritable differences in *in vitro* regenerability in *Petunia*, at the protoplast, and at the seedling stage. *In* I Potrikus, CT Harms, A Hinnen, R Hütter, PJ King, D Shillito, eds, Protoplasts. Birkhäusel, Basel, Switzerland, pp 236–237

Estruch JJ, Prinsen E, Van Onckelen H, Schell J, Spena A (1991) Viviparous leaves produced by somatic activation of an inactive cytokinin-synthesizing gene. Science **254:** 1364–1367

Fuchs R, Machleidt W, Gassen G (1988) Molecular cloning and sequencing of a cDNA coding for mature human kidney cathepsin H. Biol Chem Hoppe-Seyler **369:** 469–475

Gaspar T, Penel C, Thorpe T, Greppin H (1982) Peroxidases: A Survey of Their Biochemical and Physiological Roles in Higher Plants. University of Geneva Press, Geneva

Halperin W (1988) Attainment and retention of morphogenic capacity *in vitro*. *In* IK Vasil, ed, Cell Culture and Somatic Cell Genetics of Plants, Vol 3. Academic Press, Orlando, FL, pp 3–47

Hensel LL, Grbic V, Baumgarten DA, Bleecker AB (1993) Developmental and age-related processes that influence the longevity and senescence of photosynthetic tissues in *Arabidopsis*. Plant Cell **5:** 553–564

Holwerda BC, Galvin NJ, Baranski TJ, Rogers JC (1990) In vitro processing of aleurain, a barley vacuolar thiol protease. Plant Cell **2:** 1091–1106

Holwerda BC, Padgett HS, Rogers JC (1992) Proaleurain vacuolar targeting is mediated by short contiguous peptide interactions. Plant Cell **4:** 307–318

Holwerda BC, Rogers JC (1993) Structure, functional properties and vacuolar targeting of the barley thiol protease, aleurain. J Exp Bot **44:** 321–329

Kao FT, Hartz JA, Law ML, Davidson JN (1982) Isolation and chromosomal localization of unique DNA sequences from a human genomic library. Proc Natl Acad Sci USA **79:** 865–869

Li Y, Hagen G, Guilfoyle TJ (1992) Altered morphology in transgenic tobacco plants that overproduce cytokinins in specific tissues and organs. Dev Biol **153:** 386–395

Lohman KN, Gan S, Manomara CJ, Amasino RM (1994) Molecular analysis of natural leaf senescence in *Arabidopsis thaliana*. Physiol Plant **92:** 322–328

Lütcke HA, Chow KC, Mickel FS, Moss KA, Kern HF, Scheele GA (1987) Selection of AUG codons differs in plant and animals. *EMBO J* **6:** 43–48

Marchuk D, Drumm M, Saulino A, Collins FS (1990) Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. Nucl Acids Res **19:** 1154

Morel G, Wetmore RN (1951) Fern callus tissue culture. Am J Bot **38:** 141–143

Muller JF, Missionier C, Caboche M (1983) Low density growth of cells derived from *Nicotiana* and *Petunia* protoplasts: influence of the source of protoplasts and comparison of the growth promoting activity of various auxins. Physiol Plant **57:** 35–41

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant **15:** 473–497

Noodén LD, Letham DS (1993) Cytokinin metabolism and signalling in the soybean plant. Aust J Plant Physiol **20:** 639–653

Renaudin JP, Tournaire C, Brillat M, Teyssendier de la Serve B (1990) Sequential hormone requirement for growth and organogenesis of *Petunia hybrida* protoplasts-derived calli. Plant Sci **71:** 239–250

Renaudin JP, Tournaire C, Teyssendier de la Serve B (1991) Quantitative analysis of protein changes during meristem initiation and bud formation in protoplast-derived *Petunia hybrida* callus. Physiol Plant **82:** 48–56

Ritonja A, Popovic T, Kotnik M, Machleidt W, Turk V (1988) Amino acid sequences of the human kidney cathepsins H and L. FEBS Lett **228:** 341–345

Roberts E, Kolattukudy PE (1989) Molecular cloning, nucleotide sequence, and abscisic acid induction of a suberization-associated highly anionic peroxidase. Mol Gen Genet **217:** 223–232

Roberts E, Kutchan T, Kolakuttudy PE (1988) Cloning and sequencing of cDNA for a highly anionic peroxidase from potato and the induction of its mRNA in suberized potato tubers and tomato fruits. Plant Mol Biol **11:** 15–26

Rogers JC, Dean D, Heck GR (1985) Aleurain: a barley thiol protease closely related to mammalian cathepsin H. Proc Natl Acad Sci USA **82:** 6512–6516

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA **74:** 5463–5467

Schwartz WN, Barrett AJ (1980) Human cathepsin H. Biochem J **191:** 487–497

Sherf BA, Kolakuttudy PE (1993) Developmentally regulated expression of the wound- and pathogen-responsive tomato anionic peroxidase in green fruits. Plant J **3:** 829–833

Singh S, Letham DS, Palni LMS (1992) Cytokinin biochemistry in relation to leaf senescence. VII. Endogenous cytokinin levels and exogenous applications of cytokinins in relation to sequential leaf senescence of tobacco. Physiol Plant **86:** 388–397

Skoog F, Miller CO (1957) Chemical regulation of growth and organ formation in plant tissue culture *in vitro*. Symp Soc Exp Biol **11:** 118–140

Smart CM (1994) Gene expression during senescence. New Phytol **126:** 419–448

Smart CM, Hosken SE, Thomas H, Greaves JA, Blair BG, Schuch W (1995) The timing of maize leaf senescence and characterization of senescence-related cDNAs. Physiol Plant **93:** 673–682

Smart CM, Scofield SR, Bevan MW, Dyer DA (1991) Delayed leaf senescence in tobacco plants transformed with *tmr*, a gene for cytokinin production in *Agrobacterium*. Plant Cell **3:** 647–656

Tadeo FR, Primo-Millo E (1990) Peroxidase activity changes and lignin deposition during the senescence process in *Citrus* stigmas and styles. Plant Sci **68:** 47–56

Teramoto H, Momotani E, Takeba G, Tsuji H (1994) Isolation of a cDNA clone for a cytokinin-repressed gene in excised cucumber cotyledons. Planta **193:** 573–579

Teyssendier de la Serve B, Jouanneau JP (1979) Preferential incorporation of an exogenous cytokinin, N^6-benzyladenine, into 18S and 25S ribosomal RNA of tobacco cells in suspension culture. Biochimie **61:** 913–922

Thorpe T (1980) Organogenesis *in vitro*: structural, physiological and biochemical aspects. Int Rev Cytol **11:** 71–112

Traas JA, Renaudin JP, Teyssendier de la Serve B (1990) Changes in microtubular organization mark the transition to organized growth during organogenesis in *Petunia hybrida*. Plant Sci **68:** 249–256

Van der Berg BM, Wijsman HJW (1981) Genetics of the peroxidase isoenzymes in *Petunia*. I. Organ specificity and general aspects of the peroxidase isoenzymes. Theor Appl Genet **60:** 71–76

Watanabe H, Abe K, Emori Y, Hosoyama H, Arai S (1991) Molecular cloning and gibberellin-induced expression of multiple cysteine of rice seeds (oryzains). J Biol Chem **266:** 16897–16902

Watillon B, Kettmann R, Boxus P, Burny A (1991) Characterization of two gene transcripts modulated by cytokinins in micropropagated apple (*Malus domestica* (L. Borkh) plantlets. Plant Physiol **96:** 479–484